1808

PATENT #32
Atty. Docket No. 8636

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPELLANTS	:	David H. Gelfand et al.	
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TITLE	:	PURIFIED THERMOSTABLE ENZYME	

REPLY BRIEF UNDER 37 C.F.R. §1.193 (b)

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

The Examiner's Answer dated May 13, 1994, raises seven new points of argument in the rejection under §112 and nine new points of argument under §103 maintaining the rejections of Claims 1, 35-39, and 53-62 all appealed claims in the above-referenced application. Below, Appellants address each new point of argument. Appellants respectfully request Examiner to enter the Reply Brief into the record and reconsider and withdraw the rejections of Claims 1, 35-39, and 53-62 under 35 U.S.C. §112, §102, and §103.

The Rejection Under 35 U.S.C. §112

1. The Examiner's Reliance on Appellant's Specification to Support the §112 rejection is New; For the First Time The Examiner Relies on the Effects of Changes in pH and the Presence or Absence of Substrates to Support The Rejection

Examiner has for the first time responded to Appellants repeated requests for a reasoned explanation in support of the §112 rejection. However, the Examiner's new reasoning is not sound; it is flawed and irrelevant. At page 4 of the Examiner's Answer, Examiner offers supportive "evidence" for his conclusionary remarks asserting "unpredictability" of the claimed invention. Appellants' specification serves as the source of this evidence. The Answer states, at page 4, line 6-12:

> For example, see page 49, lines 12-16, of the specification where there is substantial variation in activity of polymerase depending on the pH. Further, see lines 17-23, on page 49 how the activity of polymerase can vary according to the presence of nucleotide (sic) triphosphates and may not be consistent with an expected value.

The Examiner's remarks, while accurate, are irrelevant and reflect a naive comprehension of protein chemistry. The argument that changes in pH will affect the activity of a polymerase, is not analogous to the substitution of one non-ionic detergent for another. If at all appropriate, pH effects are analogous to the presence or absence of a non-ionic detergent. Similarly, the effect of the presence or absence of one or more nucleoside triphosphates is not analogous to the substitution of one non-ionic detergent for another. If any conclusion regarding detergent is appropriate, and it is not, a possible conclusion could be that the presence or absence of non-ionic detergent could affect activity. A conclusion regarding the effect of substituting one non-ionic detergent for another is not reasonably based on the cited passages in Appellants' specification.

Changes in pH will directly affect the charge of amino acids within a protein's polypeptide chain. These changes are well known in the art to directly influence the secondary structure, i.e. folding, of the protein and reactivity with substrates. Consequently, changes in pH may cause changes in conformation and may therefore, directly affect the ability of enzymes to function and bind to their normal substrates.

Similarly, non-ionic detergent can affect the state of aggregation, adherence to surfaces and/or conformation, and thus, the activity of a protein. Appellants' invention is the discovery that the presence or absence of non-ionic detergents does affect the storage stability and thus the activity of a purified thermostable DNA polymerase, and that effect is critical to storage stability. Appellants have identified a class of compounds necessary for storage of the enzyme without concomitant loss of activity. The Examiner has not presented any evidence why one of skill in the art would expect the substitution of one member of the class for another member to have a different effect.

The Examiner's support for his rejection, by relying on the description that the enzyme requires four nucleoside triphosphates for activity, is a further new point of argument. However, this new argument is irrelevant. Nucleoside triphosphates (dNTPs) are substrates for the polymerase. The passage of the specification referred to (page 49, lines 17-23) describes that the purified thermostable enzyme's requirement, for all substrate dNTPs to be present in the reaction mix for significant template-dependent polymerization activity, is indicative of the enzymes high

fidelity. There is no discussion in Appellants' specification of substituting one dNTP for another, which would seem to be the closest analogous issue. Further, even if such a discussion did exist, either in the present application or the Examiner's argument, it would have no relevance to the issue at hand. Unlike dNTPs, a non-ionic detergent is not a substrate for the enzyme. There is no reasonable analogy; no conclusions can be drawn.

2. The Examiner's Argument That The Wu et al Reference May Have Shown Different Results Had Different Experiments Been Conducted Is New and Without Scientific or Legal Merit

In the Answer, at page 4, lines 12 and 13, the Examiner supports his §112 rejection by referring to his arguments under §103 concerning the Wu et al. reference (the Wu et al. reference is appended to Appellants' Brief as Exhibit F). In that argument the Answer characterizes Wu as deficient because only one non-ionic detergent is used at Table III, page 791. The Answer states:

> The Triton-X-100 detergent or other detergents used alone may not have had any effect on the DNA polymerase of the claims.

(page 22, lines 5-7) (emphasis added).

The Examiner's speculation as to what may or may not have been shown, had Wu done the Examiner's hypothetical experiments, does not render the reference deficient and cannot be taken as support for the enablement rejection. Wu is art-recognized knowledge that non-ionic detergents function equivalently. The fact that Appellants' example uses two non-ionic detergents and Wu used non-ionic detergents individually, does not mean that Wu is properly disregarded as credible evidence to overcome the rejection of the present claims.

Enablement requires that the specification teaches how to use the invention. The specification teaches that at least one non-ionic detergent is required, and Appellants teach how to add just one detergent. The Examiner may be arguing that by not exemplifying only one non-ionic detergent in a buffer, Appellants have not demonstrated that the claimed invention will work. If so, this point is not relevant for enablement. Should the issue be a question of utility, Appellants direct the Board's attention to Examiner's §102 reference, the MBR product information sheet, where the shipping and storage buffer contains a single non-ionic detergent.

Thus, the Wu reference does not provide scientific support for the Examiner's assertion. Further, the law does not hold that a rejection under the first paragraph of §112 can be properly based on what an undescribed experiment might have demonstrated.

3. The Examiner's Argument Addressing Claim 62 Is New: For The First Time The Examiner Specifically Discusses Claim 62 Under §112 and He Has Mis-read The Application

For the first time the Examiner specifically addresses Independent Claim 62 in the rejection under 35 U.S.C. §112. In his answer, at page 10 in the paragraph beginning at line 4, the Examiner raises a new and erroneous point of argument.

The Examiner's new point of argument refers to Appellants' Brief at page 12, where Appellants point out where the specification provides particular support for Claim 62. Specifically at page 10, line 13, the Answer states:

> The reaction buffer described on page 12 of the brief does not contain any non-ionic detergent.

Appellants assume that this conclusion is intended as a new point of argument to support the enablement rejection; however, the Examiner's statement is erroneous. The paragraph at page 10 of the Answer accurately describes that the reaction, described on page 79 of the specification, utilizes the polymerase which had been stored in a storage buffer and subsequently diluted into a dilution buffer, wherein both the storage and dilution buffers comprise non-ionic detergent.

This diluted polymerase composition is then added to the reaction mixture, exactly as described in Claim 62. Thus, the final reaction mixture of Example XIV, reproduced at page 12 of the Brief, contains the polymerase composition including non-ionic detergent. The specification, and even the Examiner's discussion, are both quite clear that the diluted polymerase and, therefore, the reaction mixture do, in fact, contain non-ionic detergent. Only the Examiner's conclusion, that the reaction buffer of Example XIV does not contain non-ionic detergent, is incorrect. Therefore, this new point of argument cannot support the enablement rejection, because it is not based on fact.

4. The Examiner's Demand For Comparative Testing Is New: The Demand For Testing is Not Justified

The Examiner raises a new point of argument by demanding additional experiments to demonstrate enablement under §112. The Examiner has always maintained that a single embodiment is insufficient because biochemical reactions are unpredictable. However, in the Answer at page 11, lines 12 and 20, the Examiner, for the first time, specifically describes two particular and new requirements:

(1) If a single detergent or combination of detergents other than the exemplified can be used, then representative number of detailed working examples should have been provided using a single detergent and other combination of detergents

(2) If glycerol, Tris-HCl pH 8.0, ethylenediaminetetraacetic acid [EDTA], dithiothreitol [DTT], and gelatin are not critical and can be omitted, then a representative number of detailed working examples should have been presented showing results when these components are omitted.

In response to demand (1) Appellants assert that the demand is without merit under the law because the Examiner has not provided any references that suggest that substitution of one non-ionic detergent for another, or substitution of one non-ionic detergent in place of two are shown to have any different effect on stabilizing a purified thermostable DNA polymerase. Without such evidence there is no need for Appellants to provide more than the specification as originally filed provides: one detailed example and a list and chemical description of equivalents.

Further, Appellants assert that the Examiner's view of a "representative number" of experiments is vague at best and provides no guidance for Appellants in their quest for a reasonable discourse and resolution of the issues on appeal.

The references in the record (see Appellants' Brief at Exhibits A, F and G) as well as the storage buffers of commercially available purified thermostable polymerases support Appellants assertion that the demand is inappropriate. "In the absence of any apparent reason why the compounds disclosed will not so function, or any evidence showing that they actually do not, the statements in the application are generally deemed sufficient." In re Chilowsky, 108 USPQ 321 (CCPA 1956).

In response to demand (2) Appellants assert that the demand is without merit because several of the suggested experiments have been previously conducted and those results provided to the Examiner and made of record in Declarations under 37 C.F.R. §1.132. Further, the demand is without merit because the Examiner has shown no evidence to support the implicit assertion that Appellants have misunderstood their own data and mistakenly identified a critical component of their invention.

The supporting laboratory notebook pages appended to the 132 Declaration made of record as Paper No. 26 (Exhibit C of Appellants' Brief) and discussed in the Declaration at items 10 - 12, document that the polymerase had been stored in a buffer with and without gelatin, and with and without non-ionic detergent. That evidence demonstrates and the Declaration states that the composition stored without gelatin did not lose activity. The inventors concluded that the presence of non-ionic detergent prevented activity loss. Thus, a careful reading of the evidence already of record directly addresses one of the four items listed in the Examiner's demand. The evidence demonstrates that gelatin is not critical and can be omitted.

The Examiner's new demands suggest that the Examiner is concerned that the invention would not work with only a non-ionic detergent and a thermostable DNA polymerase, and that this composition would be within the scope of the claims. Appellants' specification teaches those of skill in the art what the invention is and how to practice it. Additional components such as glycerol to prevent freezing and Tris-HCl for maintaining a desired pH, as well as EDTA and DTT are widely known in the art of buffer formulations and are well within the scope of general knowledge of the ordinary artisan. Substitutes for these components, such as EGTA for EDTA, are known equivalents. There is no evidence presented for supporting the Examiner's demand for testing his assertion that these agents must be included in the claims.

The Examiner's ability to envision a clearly non-applicable scenario is not a proper basis for a rejection of the claims. As stated by the C.C.P.A.:

> [i]t is not a function of the claims to specifically exclude either the possibility of inoperative substance or ineffective reactant proportions.

In re Ding-Nguyen and Stenhagen, 181 U.S.P.Q. 46 (CCPA 1974). The possibility that the claims encompass a non-working embodiment either by choice of non-ionic detergent or by exclusion of a buffering agent is insufficient "evidence" of non-enablement. As further stated by the C.C.P.A.:

> it is possible to *argue* that process claims encompass inoperative embodiments on the premise of unrealistic or vague assumptions but that is not a valid basis for rejection.

(emphasis in original) In re Geerdes, 180 U.S.P.Q. 789 (CCPA 1974).

5. The Examiner's Argument, That Different Non-ionic Detergents Differ Substantially in Structure and Chemical and Physical Properties, Is New

For the first time the Examiner's supplies his detailed reasoning for asserting that the guidance provided by the specification at page 24 is insufficient. At page 12, lines 3-9, of the Answer the Examiner explains his views:

> The protein structure can be easily denatured by conditions that break bonds that hold the structure together and result in uncoiling or unraveling of the structure . . . [D]ifferent non-ionic detergents can differ substantially in structure and chemical and physical properties. Such different non-ionic detergents would not be expected to affect the activity of an enzyme the same.

The Examiner's specific argument is new and Appellants respectfully traverse. Denaturation by detergent is well known. Differences between types of detergents, such as non-ionic, anionic and zwitterionic detergents are also well known to those of ordinary skill possessing a basic biochemistry or chemical background. However, the specification teaches and claims a defined class of non-ionic detergents (see page 24 beginning at line 21 in the section entitled "Stabilization of Enzyme Activity").

The Examiner's new assertions that different non-ionic detergents would not be expected to affect the activity of an enzyme in the same manner, is not supported and is not supportable. It is an erroneous conclusion that apparently exists only in the Examiner's mind. The Examiner has never provided any evidence for supporting his assertion that substitution of one non-ionic detergent for another is not expected to provide a workable invention. Without some evidence contradicting the teaching of Appellants' specification, the rejection is improper and must be reversed.

6. The Examiner's Application of His Arguments Under 35 U.S.C. §103 to Support His Rejection Under §112 Is New

At page 13 of his Answer the Examiner refutes Appellants' arguments traversing the rejection under §112 and relies on his earlier remarks in the Office Action dated July 15, 1992, at pages 5 and 6 (Paper No. 21). Surprisingly, the remarks at pages 5 and 6 of that Action are in the section explaining the Examiner's rejection of the claims under §103. Clearly, the Answer at page 13, beginning at line 5, raises new points of argument by relying on a discussion of a §103 rejection for supporting the present rejection under §112.

In this section of his Answer, the Examiner addresses the Rule 132 Akers Declaration. The data presented in the Akers Declaration was intended to traverse the Examiner's obviousness rejection under §103. The Examiner now is attempting to discredit the experimental evidence provided by Akers based on a purported insufficiency for demonstrating enablement of the scope of the claims. See for example, the Answer at page 13, lines 11-13. This new point of argument should be recognized for what it is: a new ground of rejection.

The Examiner for the first time points out that the experiments provided by Akers use a reverse transcriptase at Experiment 1 and a DNA polymerase at Experiments 2 and 3. In asserting that the Akers Declaration supports his rejection, the Examiner asks whether these were "intended to be different enzymes or the same enzyme" (see the Answer at page 14, lines 12-13).

Appellants find that the question posed by the Examiner demonstrates that the Examiner has never read nor comprehended Appellants' data or his own remarks in the record. The Examiner has inappropriately waited until preparing his Answer to request clarification of data placed in the record over two years ago.

The basis for Examiner's obviousness rejection was his assertion that reverse transcriptase and thermostable DNA polymerase are analogous enzymes and that the behavior of reverse transcriptase would predict the behavior of purified thermostable DNA polymerases. Appellants traversed the rejection by providing experimental data demonstrating that there is no analogous effect of non-ionic detergent on reverse transcriptase and purified thermostable DNA polymerase. The experimental data supports this conclusion and serves to refute the Examiner's obviousness rejection.

The presentation of these arguments in support of the rejection under §112, first paragraph, is not only a new argument, it is an absurd argument.

7. The Examiner's Use of Kaledin et al (1980) to Interpret Example XIV of the Specification is New

At page 14, line 4, of the Answer, the Examiner refers to Kaledin's use of gelatin as a stabilizer and notes that Kaledin does not show results when gelatin is used with a non-ionic detergent. The Examiner compares the 1980 reference to the specification at Example XIV where gelatin is present with non-ionic detergent in the enzyme storage buffer. The Examiner concludes at page 14, line 8:

> Since this [Appellants'] example uses this combination, apparently the combination was found to provide better results.

This suggestion, that in view of Kaledin, the specification incorrectly describes the invention, is a new point of argument based on an erroneous conclusion. The Examiner is now wandering into the realm of questioning the materials and methods included in Appellants' experimental design at the time the present application was filed. The Examiner then proceeds to answer his hypothetical questions based on unsupported hindsight and non-comparable data (i.e. Kaledin et al. (1980) and the present application).

Appellants have provided declarations under Rule 132 and Rule 131 demonstrating that gelatin does not provide enhanced stability or activity for a purified thermostable DNA polymerase. Present commercial embodiments of the invention do not include gelatin in the enzyme storage buffer. Even the subsequent publication of Kaledin et al. (1981) describes that gelatin is not required for stabilization.

The Examiner has ignored the references, ignored the specification, ignored the supplemental declarations and contradicted Appellants' original description of the invention. All this has been done with no evidence to support his subjective and erroneous assertions. Accordingly, Appellants respectfully request the Board to reverse the rejection of Claims 1, 35-39 and 53-62 under 35 U.S.C. §112, first paragraph.

The Rejection Under 35 U.S.C. §103

1. The Examiner's Reasoning for Dismissing the Rule 132 Akers Declaration is New: For The First Time The Examiner Questions the Distinction Between an RNA-Dependent Viral Non-Thermostable Reverse Transcriptase and a DNA-Dependent Bacterial Thermostable DNA Polymerase

At page 20, line 5, of the Answer the Examiner states:

> The declaration fails to disclose the source of the reverse transcriptase and how it differs from the DNA polymerase in Experiments 2 and 3.

The Rule 132 Akers Declaration was first submitted to the Examiner and made of record on April 24, 1992. Yet now, after over two years of ex parte prosecution the Examiner, for the first time, asks how the reverse transcriptase of Experiment 1 is different from the purified thermostable DNA polymerase of Experiments 2 and 3 (see the Answer at page 20, line 5). The Examiner implies in his Answer that reverse transcriptase and DNA polymerase are not clear and distinct terms in the art, universally recognized as distinct types of enzymes.

Appellants find the late entry of these remarks into the record to be an unequivocal and preposterous misuse of the proceedings. Appellants' record has continually described, in accord with the totality of the references in the record, that reverse transcriptase is a term referring to viral non-thermostable enzymes that catalyze the combination of nucleoside triphosphates to a form a nucleic acid strand complementary to an RNA template. The claimed invention involves, and the enzyme of Experiments 2 and 3 is, a DNA polymerase, well known in the art as a non-viral thermostable polymerase that requires a DNA template.

The enzyme of Experiment 1 is the Moloney Leukemia Virus, non-thermostable reverse transcriptase that is included in the Roche RNA-PCR kit. This kit is specifically mentioned in the Declaration at Experiment 1. Thus, although the Declaration does not expressly identify the enzyme, in contrast to Examiner's assertion, the source of the enzyme is provided. Had the Examiner expressed this confusion during the past two years of prosecution, Appellants would have readily produced the product insert, which is now attached as Exhibit A.

The product insert evidences the factual basis that has been argued throughout the prosecution. M-MuLV-RT, the tested enzyme of Experiment 1, is Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV-RT). The enzyme of Experiments 2 and 3, as the Declaration specifically describes in the title of Experiment 2, is AmpliTaq®, and is referred to throughout the Declaration as Taq DNA polymerase. The Examiner is by now well aware that Taq

DNA polymerase, a bacterial thermostable DNA polymerase, is a preferred embodiment of present invention and has been patented (see U.S. Patent Nos. 4,889,818 and 5,079,352). Appellants note that the present application is related as a continuation-in-part to the '818 patent and a continuation of the '352 patent.

The Examiner's question demonstrates a disappointing lack of understanding in the field of the invention. As those of ordinary familiarity in the art are well aware, there are many types of enzymes that are capable of polymerizing nucleotides, including RNA-dependent viral enzymes, mesophilic DNA-dependent bacterial enzymes, DNA-dependent mammalian enzymes, RNA polymerases, RNA replicases, polynucleotide phosporylases, and terminal transferases. Scanning any table of contents in any commercial biochemistry reagent catalogue evidences that the Examiner's issue is a non-issue. Where enzymes are indexed by function, DNA polymerases are listed separately from reverse transcriptase. See, for example, the tables of contents attached hereto as Exhibit B which are taken from current copies of the major suppliers of molecular biology reagent catalogues.

To further address the Examiner's new point of argument, Exhibit B includes a graphic description from the New England Biolabs molecular biology reagent catalogue. The graphic is a table entitled "Applications of Modifying Enzymes." The table provides clear and convincing evidence, understandable by the ordinary artisan, or the unskilled layman, that reverse transcriptase and DNA polymerase are not the same enzyme but are chemically and functionally distinct classes of biomolecules.

M-MuLV-RT is a viral non-thermostable RNA-dependent enzyme that utilizes an RNA template. This enzyme is called a "reverse transcriptase," indeed because it provides a reverse transcript of an RNA target. Reverse transcriptases are purified from RNA viruses which replicate by mechanisms biologically distinct from cellular organisms. The replication processes of RNA retroviruses require an enzyme with this template specificity. The term "DNA polymerase" refers in the art to enzymes such as AmpliTaq®, a bacterial, thermostable, DNA-dependent DNA polymerase. The claimed invention relates to purified thermostable DNA polymerases. The Declaration under 37 C.F.R. §1.132 of Gelfand makes of record that which the artisan of ordinary skill in the art recognizes as fact, that reverse transcriptase and thermostable DNA polymerases are dissimilar, non-analogous, non-comparable, and not predictive of one another's properties and characteristics.

During prosecution Appellants provided Rule 132 Declarations and copies of commercial reagent suppliers' catalogue descriptions to demonstrate that, in the art, a reverse transcriptase is not analogous with the enzymes of the invention. Further, Appellants have presented evidence that, even following the Examiner's faulty reasoning, the more appropriate standard for comparing Appellants' invention would be purified non-thermostable bacterial DNA-dependent polymerases, rather than viral RNA-dependent reverse transcriptase. There are many commercially available non-thermostable bacterial polymerases, and Appellants have provided evidence that these reagents do not require non-ionic detergent for storage stability (see Appellants' Brief at Exhibit F).

2. The Examiner's Argument, that the Absence of Detergent in The Kaledin Publication Invalidates Appellants' Data Because the 132 Declaration Utilizes the "same DNA polymerase," is New

Appellants have previously argued that Kaledin's data is not predictive of the storage stability for purified thermostable polymerases. However, now the Examiner asserts not only that Appellants' data must be ignored because it refutes Kaledin, and that Kaledin is predictive of the claimed invention, but that the enzyme of Kaledin and AmpliTaq®, the enzyme of the Akers' Experiments 2 and 3, are the same (see the Answer at page 20, lines 13-18). The Examiner is mistaken and the error must be pointed out in the record.

The Kaledin enzyme is a crude preparation, comparable in activity to 1.5% of the purity of Appellants' teaching. The Kaledin reference and the present specification provide data for making this comparison. The U.S. Patent Office has previously clarified that the Kaledin preparation is not Appellants' enzyme and that the publication does not provide Appellants' purified thermostable enzyme preparation which is used in the experiments discussed in the Akers Declaration. The issuance in 1989 of the great grandparent application as U.S. Patent No. 4,889 818 and in 1992 of the grandparent application as U.S. Patent No. 5,079,352 over Kaledin (1980 and 1981) evidence the facts for the record. The Kaledin enzyme is not the enzyme of the Akers' experiments. Kaledin's results show nothing regarding the claimed formulation components.

3. The Examiner's Argument, that Experiment 1 of The Akers Declaration is Inconsistent With Experiments 2 and 3, is New

The Examiner at this late date in prosecution, has taken a new approach for attempting to defeat Appellants' evidence. He asserts in the Answer at page 20, lines 18-22, that since the Experiments of the Akers Declaration all utilize polymerizing enzymes, he can conclude that differences between reverse transcriptase (at Experiment 1) and AmpliTaq® (at Experiments 2 and 3) demonstrated by Appellants' data are inconsistent. The Examiner has built a straw man, and Appellants must traverse.

The Akers Declaration and the entire record are very clear as to how Experiments 1, 2, and 3 differ. Experiment 1 and Experiments 2 and 3 differ in enzyme and substrate. Although both use polymerizing enzymes, the difference in template dependency between reverse transcriptase and DNA polymerase required that different substrates were employed in Experiment 1 and Experiments 2 and 3. These experiments test a viral reverse transcriptase utilizing an RNA template in comparison to a purified thermostable DNA polymerase with a DNA template. The results are discussed in the Declaration and in Appellants' responses and Appeal Brief. Appellants point out that Wu supports the conclusion presented in the Akers Declaration. Further, the Examiner acknowledges the distinction between detergent stimulation of viral reverse transcriptase and expectations of an effect on bacterial DNA polymerase (see Examiner's Answer at page 22, line 18-20).

The Examiner at page 20, lines 7-10, and page 20, lines 18-20, suggests that the references cited in the rejection support his argument that reverse transcriptase and bacterial DNA polymerases are the same enzymes. This fallacious conclusion permits him to describe the Akers Declaration as inconsistent. However, even the very references he cites for support serve to illustrate the absurdity of this new argument. For example, Spiegelman describes a novel viral like polymerase purified from breast tumor tissue. At column 2, lines 24-36, the reference provides a detailed description of key properties that are used to distinguish viral reverse transcriptase from normal mammalian DNA polymerases (see especially line 29).

The Examiner's reliance to Goff at column 8, lines 16-18, raises a new point of argument (see the Answer at page 20, line 9). Goff describes cloned viral reverse transcriptase, as well as expression and characterization of the engineered products. The paragraph at column 8, line 16, discusses the measurement of reverse transcriptase activity in the recombinant cultures. The

sentence referred to by the Examiner actually begins at line 15 and must at least be viewed in the context that a full sentence provides:

> The bulk of this background activity is attributable to the presence of DNA polymerase I in the extracts; this enzyme is known to exhibit reverse transcriptase activity.

Thus, the statement relied on for supporting the Examiner's position that DNA polymerase and reverse transcriptase are not distinguishable in the art, actually serves as clarification. Goff finds that DNA polymerase is present as a contaminant; thus, it is distinguishable from the enzyme of interest, reverse transcriptase. In stating that the DNA polymerase may have reverse transcriptase activity indicates that the activity of a DNA polymerase is not the activity of a reverse transcriptase. These are indeed different, distinguishable enzymes.

The Examiner's argument is based on his own invented use of these terms. They are ordinary terms of art and the recognized meanings are well known and supported by the numerous references and reagent catalogue descriptions in the record. The Akers Declaration is only inconsistent with the Examiner's subjective basis for rejecting the claims.

4. The Examiner's Argument, that the Akers Declaration Must Show Storing the DNA Polymerase for Different Periods of Time, is New

The Examiner has for the first time argued that the Akers Declaration is insufficient because it does not show storing the enzymes for different periods of time prior to testing (see the Answer at page 21, lines 3-5). Appellants again must question why the Examiner has waited until this late date, fully two years after the Rule 132 Declaration was made of record, to raise this new point of argument. Appellants must point out that given the time period for response, a further Declaration that meets the Examiner's new criteria for long term storage is not possible under the law.

Further, Appellants respectfully assert that the Examiner's demand is without merit. There is no suggestion in the specification or claims that there are particular time parameters for judging stability of the claimed formulation. The experiments provided in the specification as well as in Akers Declaration offer ample demonstration of the need for non-ionic detergent in the storage buffer for purified thermostable polymerase. Should the Examiner intend to suggest that once lost, catalytic activity would return upon extended storage, Appellants would point to the laboratory

notebook experiments provided in the Rule 131 Declaration that describe the effects of long term storage in the presence and absence of non-ionic detergent (see Appellants' Brief at Exhibit C, beginning at paragraph 10).

Apparently, the Examiner missed the point of the Akers Declaration. The Declaration served to refute the Examiner's position that viral non-thermostable RNA-dependent reverse transcriptase is predictive of purified bacterial thermostable polymerase (see the Office Actions dated September 4, 1990, at page 6, lines 20-26, and May 3, 1991, at page 5, lines 9-23). The Akers Declaration further demonstrates that in contradiction to the Examiner's conjectural arguments, gelatin is ineffective for providing a storage stable thermostable enzyme composition.

5. The Examiner's Argument, that the Akers Declaration Must Show Results From Experiments Combining Gelatin With NP-40 and Tween-20, is New

Again, Appellants regret that two years after the Examiner had first considered the Akers Declaration, the Examiner now argues for the first time that additional experiments should have been included. Specifically, the Examiner states that because the specification exemplifies a buffer comprising gelatin and non-ionic detergent, that buffer must be included in the Rule 132 Declaration (see the Answer at page 21, lines 5-8). Appellants are not aware of such a standard.

The Rule 132 Akers Declaration compares the claimed invention to the closest prior art in view of the rejection. Throughout prosecution of the present claims the Examiner has repeatedly argued that gelatin and non-ionic detergent were obvious equivalents for stabilizing the purified thermostable polymerase (see, for example, the Office Action dated May 3, 1991, at page 5, lines 11-16). The Akers Declaration refutes that assertion.

In addition to the Akers Declaration, the notebook exhibits that accompany the Rule 131 Declaration (Exhibit C of Appellants' Brief) demonstrate that at the time of filing the Akers Declaration, Appellants were well aware that gelatin had no additional effect for stabilizing the purified enzyme in the presence of non-ionic detergent. The present commercial embodiments of the invention, as well as competitors' copying of the invention do not include gelatin in their storage buffers. Again, the descriptions of major reagent suppliers storage buffers demonstrate that the Examiner's newly proposed experiment is without merit, is requested in an untimely fashion, misses the point of the Rule 132 Declaration, and ignores the laboratory notebook evidence already of record.

6. The Examiner's Interpretation of the Prior Art Concerning the Requirement for Detergent is A New Point of Argument

The Examiner, for the first time, asserts that it is known in the prior art that the enzyme does not require a detergent to function (see the Answer at page 13, lines 11-16). Appellants cannot let this bold statement, presenting a new point of argument, go unanswered. However, the Examiner's assertion is difficult to respond to, because the remark is unclear as to what is meant by "the enzyme" at line 16. The Examiner seems to have confused reverse transcriptase with thermostable polymerase. In the context of the argument he has set forth, the sentence may intend "the enzyme" to refer to either the reverse transcriptase of Experiment 1 or the thermostable DNA polymerase of Experiments 2 and 3. The Examiner refers to the "prior art" without specific support that would assist Appellants in understanding the Examiner's intent. The inability to clearly and unambiguously state his objections and correctly summarize the prior art places an unreasonable burden on Appellants.

At page 19, lines 18-22, the Examiner relies on his conclusory remarks about the prior art and "the enzyme" in arguing that the 132 Akers Declaration is without merit:

> Appellants assert that the 132 Akers Declaration shows that reverse transcriptase functions equivalently in the presence or absence of the non-ionic detergent, NP-40. However, as stated above, it is known that the detergent is not needed for the enzyme to function.

Again, it is not clear which enzyme the Examiner intend to discuss. Indeed, by "the enzyme" the context suggests that "reverse transcriptase" is intended. However, by this interpretation, the statement would contradict for the first time, the Examiner's previous position that the requirement for non-ionic detergent in a reverse transcriptase buffer for purification obviates the present invention. The Examiner's remark and intended meaning are unclear. In view of his characterization of the prior art (i.e., "it is known that . . .") without citing to the particular art relied upon, Appellants request reversal of the rejection.

7. The Examiner's Argument, that the Gelfand Declaration is Not Sufficiently Supported is New; The Requirement for Setting Forth the Particular Amino Acid Sequence Differences Between Taq and M-MuLVRT, is New

The Rule 132 Gelfand Declaration was made of record in April 1992. In the past two years the Examiner has acknowledged that reverse transcriptase and Taq polymerase differ in amino acid sequence as described in the Gelfand Declaration. Throughout the prosecution the Examiner has asserted that the differences are not relevant.

Now, for the first time, the Examiner argues that the Declaration does not adequately describe experiments or procedures that led to the asserted conclusions. Specifically, at page 21, line 14, the Examiner's Answer states: "[H]ow the amino acid sequences differ has not been specifically set forth." In view of his new requests for additional information, Appellants will directly address the Examiner's issues below.

The Gelfand Declaration analogizes the prior art in an appropriate way. The enzyme exemplified in the specification was compared to the reverse transcriptase that is the subject of Goff. Specifically, the complete sequence of the Moloney Murine Leukemia Virus genome was retrieved from the GenBank database (locus designation: MLMCG). This sequence was deposited in the data bank on December 15, 1989, and was first published in 1981 (e.g., Nature 293:543-548). The portion of the 8,332 nucleotide sequence which corresponds to the M-MuLV Reverse Transcriptase coding region of Goff was translated into a 664 amino acid protein.

The Examiner's new points of argument are without merit. The amino acid sequence of the purified thermostable DNA-dependent polymerase from Thermus aquaticus is provided in the present specification. The amino acid sequence of Moloney Murine Leukemia virus reverse transcriptase is provided by the Examiner's reference of record, Goff (ATCC Deposit No. 39939), and has been published. As described above, this sequence is readily available from GenBank, a widely available data base of published nucleic acid sequence information. Thus, Appellants used the closest art, at least according to the Examiner, for the purpose of comparison.

The Gelfand Declaration describes that a computer program was used to compare the two published sequences. A number of programs are currently available and the choice of program cannot change the facts. The "Wisconsin Package" of the Genetics Computer Group (GCG) contains a program entitled "GAP" which is frequently used to look for relatedness between two sequences and also to attempt to align the sequences. The GAP program always produces an alignment and a "Quality Score." To assess the significance of the alignment and the "Quality

Score," the program provides an optional "Randomization" feature. This "Randomization" feature scrambles or randomizes the precise first amino acid sequence while maintaining the exact amino acid composition. Each of these randomized test sequences is aligned with the second specific sequence and a "Quality Score" is generated. Clearly, if two proteins are significantly related, then the "quality score" of the specific alignment would be significantly greater than the "quality score" based on the alignment of random sequences (see the Gelfand Declaration at paragraph 4, Exhibit E, of Appellants' Brief).

The Gelfand Declaration states that the MuLV RT of Goff and Taq DNA polymerase are not related and that the degree of relatedness between the two proteins was worse than for comparisons with the random sequences. The amino acid sequences are unrelated. The Examiner's argument, that how the amino acid sequences differ has not been set forth, is irrelevant. However, in response to the Examiner's belated request, and so that the Board may understand that a computer-generated alignment between two unrelated sequences has no significance, other than showing that the sequences are not related, the alignments carried out by Dr. Gelfand in January, 1992, are attached hereto as Exhibit C.

As stated in Dr. Gelfand's Declaration, and demonstrated in the accompanying computer print outs, the thermostable polymerase and the viral reverse transcriptase have a quality score of 220, whereas the thermostable DNA polymerase and E. coli Pol I have a quality score of 609. These results indicate that the thermostable bacterial polymerase is more closely related to E. coli Pol I than to the viral reverse transcriptase. The comparison to prior enzymes for determining the patentability of the claimed invention must be made with the closest prior art and not the Examiner's conjectural models.

Appellants suggest that scientific reasoning should determine the merits of the case and not the Examiner's quest for the purportedly missing evidence that would support his, thus far, unsubstantiated theorizing.

8. The Examiner's Argument, that Wu Discloses Stimulation is Dependent on the Amount and Type of Template-Primer but that Stimulation is Seen in the Absence of Template-Primer, is New

The Examiner's Answer (page 22, lines 15 and 16) accepts and acknowledges one of the clear conclusions of Wu: "While Wu et al. disclose that stimulation is dependent on the amount and type of template-primer . . ." However, the Examiner's assertion in the rest of that sentence

(page 22, lines 16-18): ". . . in Table I it appears that stimulation is obtained without the presence of template-primer" is new and completely incorrect. The legend to Table 1 (Wu et al., page 790) clearly states: "The procedure for measuring $(dT)_n$ synthesis was the same as described under Materials and Methods." Appellants assert that any reader would know to consult the "Materials and Methods" section of the publication for additional details (pages 789-790). Indeed, the pertinent portion of that section is immediately below Table 1. In this section, Wu et al. describe in detail how they measure $(dT)_n$ synthesis. The description states that the assay uses "10μg/ml of $(dT)_{12\text{-}18}$•$(rA)_n$" indicating that a short (12 to 18 nucleotides long) oligo-deoxythymidine primer is hybridized to a long RNA template of repeating rAMP residues. Thus, even a cursory reading of the legend to Table 1 and the materials and methods teaches that, in contrast to Examiner's remarks, template-primer is present.

The statements reproduced above from page 22 of the Answer reflect a lack of understanding of the field, the cited literature and the Examiner's own previous arguments. Unfortunately, this shortcoming leads to repeated mis-statements of fact introduced by the Examiner and placed in the record. Appellants note this new point of argument to clarify and distinguish for the record that which is present in the prior art from that which is envisioned by the Examiner.

9. The Examiner's Argument, that Wu Indicates Unpredictability and Supports the §112 Rejection, is New

Appellants noted above, at Section 6 in response to the Examiner's new points of argument under §112, that arguments of obviousness are inappropriate and irrelevant when presented in the Examiner's explanation of support for the enablement rejection. Similarly, in his Answer in the section entitled "Arguments to Rejections Under 35 U.S.C. §103," the Examiner cannot fairly introduce a new point of argument for supporting the §112 enablement rejection. Yet at page 22, line 11, this is exactly what the Examiner attempts. The Examiner's confusion regarding the distinction between obviousness and enablement serves only to compound the difficulty of preparing an appropriate reply for review by the Board. Appellants regret any confusion that results; however, this new point of argument must be addressed, and Appellants will do so in the order in which the Examiner's new point was raised. Consequently, the merits of this new argument regarding enablement, raised under §103, will be traversed for the record, albeit briefly.

Specifically, for the first time the Examiner argues at page 22, lines 11-15:

> Wu et al. provides support for the 112 breadth rejection since this reference indicates unpredictability and that certain detergents within the scope of the claims will not stabilize bacterial DNA polymerase.

The Examiner has failed to grasp the relevance of the reference or even to understand the experiments designed and explained by Wu. The argument, used by the Examiner in the section entitled "Arguments to Rejections Under 35 U.S.C. §112," was that the scope of the claims was not supported because of the unpredictability of non-ionic detergent substitutions. In the passage of the Answer reproduced above the Examiner asserts that Wu supports this rejection based on "unpredictability." The reference, however, does not demonstrate unpredictability of one detergent substituted for another.

Wu's analysis teaches that non-ionic detergents are interchangeable equivalents. See Table 1 at page 790 and the text at the end of col 2 of that page where the reference states that the non-ionic detergents tested have similar stimulatory activity. If there is unpredictability within the class of non-ionic detergents, the Examiner has yet to provide any evidence to support his hypothesis.

The Examiner's new point of argument serves to demonstrate a second assertion which Appellants have repeatedly presented and provided evidence to support; non-thermostable bacterial DNA polymerases do not require non-ionic detergent for storage stability. Indeed, Appellants agree with the Examiner: non-ionic detergents do not stabilize non-thermostable bacterial DNA polymerase (see the Answer at page 22, lines 13, 14, and 18-20). The prior art non-thermostable bacterial enzymes are not within the scope of the claims but they do represent the closest art.

Appellants respectfully assert that the Examiner's remarks demonstrate that the prior art teaches away and the claimed invention is not at all obvious, but is in fact surprising and unexpected. Appellants request reconsideration and reversal of the rejection of Claims 1, 35-39, 53-62 under 35 U.S.C. §103.

Conclusion: The Invention Is Not Incredible

The Examiner argues that the invention is incredible. (The Answer at page 9, line 14). The Examiner further argues that the invention is obvious. The invention has been copied by all competitors in the trade. Prior to Appellants' invention suppliers of DNA-dependent nucleic acid polymerase did not include non-ionic detergent in their storage buffers. At that time, purified

thermostable DNA polymerases were unavailable. Today, the major suppliers of purified thermostable nucleic acid polymerases, who provide this information in their catalogues, utilize the formulation claimed in the present case on appeal.

Based on the arguments presented above and in Appellants' Appeal Brief, and the evidence already of record in the present application Appellants submit that the rejections under 35 U.S.C. §112, §102, and §103 are not supported by the law, the Examiner's reasoning or the references. Therefore, Appellants respectfully request that the final rejection of all appealed claims be reversed.

The Commissioner is hereby authorized to charge any fees in connection with this Reply Brief to Deposit Account No. 08-2525.

Respectfully submitted,

By: 

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PERKIN ELMER CETUS
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GeneAmp® RNA PCR Kit

NOT FOR DIAGNOSTIC OR MEDICAL USE
FOR RESEARCH USE ONLY
See notice to purchaser

Part No. N808-0017

The Perkin-Elmer Cetus *GeneAmp*® RNA PCR Kit is designed for use in the detection and analysis of gene expression at the RNA level. Included are reagents to perform the reverse transcription of RNA to cDNA (using cloned Moloney Murine Leukemia Virus [M-MLV] Reverse Transcriptase) and subsequent amplification (using the *GeneAmp*™ Polymerase Chain Reaction [PCR] process and *AmpliTaq*® DNA Polymerase) all in a single reaction tube.[1-3] A recombinant RNase inhibitor, originally isolated from human placenta, is included for inhibition of certain mammalian RNases.[4] Sufficient reagents are provided for 100 reverse transcription reactions (20μL each) and 100 PCR amplifications (100 μL each).

The kit also contains a Positive Control RNA template transcribed from the plasmid pAW109. Plasmid pAW109 contains an insert of a synthetic linear array of primer sequences for multiple target genes constructed such that "upstream" primer sites are followed by complementary sequences to their "downstream" primer sites in the same order. The insert in pAW109 is identical to the insert previously described for plasmid pAW108.[1] The primers included in this kit flank an IL-1α site and can be used to amplify a 308 bp sequence within the site. Sufficient Positive Control RNA is provided for 25 reverse transcription/amplification reactions at 10^4 copies per reaction.

The *GeneAmp* PCR process is a simple and powerful method,[5] invented by K. Mullis and patented by Cetus Corporation, which allows *in vitro* amplification of DNA segments (including cDNA)[1-3] through a succession of incubation steps at different temperatures.[6-9] The *GeneAmp* PCR process is based on the repetition of this cycle and can amplify DNA segments by at least 10^5 fold, and potentially as high as 10^9 fold,[5] under the conditions described below.

LIST OF KIT COMPONENTS

Store the *GeneAmp* RNA PCR Kit at -20°C in a constant temperature freezer. If stored under the proper conditions, the product will maintain performance until the control date printed on the label.

Important Note: **Reverse Transcriptase and RNase Inhibitor are sensitive to air oxidation. Keep these reagents at -20°C until just prior to use.**

Reagent	*Volume*	*Concentration*	*Comments*
Reverse Transcriptase	100 μL	50 U/μL	Reverse transcribes RNA to cDNA
RNase Inhibitor	100 μL	20 U/μL	Inhibits RNase activity
Random Hexamers	100 μL	50 μM*	Random primers for reverse transcription of RNA
Oligo $d(T)_{16}$	100 μL	50 μM*	Poly (rA) tail primer for reverse transcription
Primer DM152	50 μL	15 μM*	"Downstream" IL-1α primer for reverse transcription and PCR of Positive Control RNA
Primer DM151	50 μL	15 μM*	"Upstream" IL-1α primer for PCR of Positive Control Template RNA
Positive Control RNA	50 μL	5×10^3 copies/μL	RNA transcribed from pAW109; in 30 μg/mL *E.coli* rRNA; in 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 8.0
AmpliTaq DNA Polymerase	50 μL	5 U/μL	Extends primers during PCR amplification
dATP	320 μL	10 mM	Deoxynucleosidetriphosphates dissolved in glass distilled water; titrated with NaOH to pH 7.0
dCTP	320 μL	10 mM	
dGTP	320 μL	10 mM	
dTTP	320 μL	10 mM	
10X PCR Buffer II	1.4 mL	500 mM KCl, 100 mM Tris-HCl	Provides preferred pH (8.3) and ionic strength for reverse transcription and PCR amplification
$MgCl_2$ Solution	1.4 mL	25 mM	Required for enzyme activity

* Supplied in 10 mM Tris-HCl, pH 8.3.

NOTICE TO PURCHASER ABOUT LIMITED LICENSE

The reagents contained in this kit are designed for use in the *GeneAmp* Polymerase Chain Reaction (PCR) process covered by patents issued to Cetus Corporation. The seller has a limited license under such patent rights, and purchase of this product includes a fully paid-up, limited, non-exclusive sublicense under such patent rights to use this product to perform the *GeneAmp* PCR process only for research and development activities and for industrial quality assurance testing. For this purpose the license for "research and development" includes the development of commercial products, such as new therapeutic products. No license is granted for any other uses, for example (1) the diagnosis or detection of disease or other health-related condition in humans, animals, or plants, or (2) genetic analysis for identification of individuals for forensic or other purposes. No other license is granted expressly, impliedly, or by estoppel. For information concerning the availability of additional licenses to practice the PCR process, contact Cetus Corporation, Director of Business Development, at 1400 Fifty-Third Street, Emeryville, CA 94608, Tel. (415) 420-3300.

AmpliTaq® Recombinant Taq DNA Polymerase
(Deoxynucleosidetriphosphate: DNA Deoxynucleotidyltransferase, E.C.2.7.7.7)

The key component in the *GeneAmp*® RNA PCR Kit is *AmpliTaq* DNA Polymerase **(Part No. N801-0060)**. Perkin-Elmer Cetus *AmpliTaq* DNA Polymerase is a recombinant, thermostable, 94kDa DNA polymerase encoded by a modified form of the *Thermus aquaticus* DNA polymerase gene[10] which has been inserted into an *Escherichia coli* host. *AmpliTaq* DNA Polymerase and native *Taq* DNA Polymerase are covered by U.S. Patent 4,889,818 issued to Cetus Corporation, and licensed to Perkin-Elmer Cetus Instruments. Patent applications are pending for genes encoding thermostable DNA polymerases, in addition to methods and formulations for stable preparations of thermostable DNA polymerases.

PROTOCOL FOR REVERSE TRANSCRIPTION OF RNA AND PCR AMPLIFICATION OF cDNA

1.0 Reverse Transcription Protocol

1.1 Prepare a master mix by adding the reagents in the order and proportions shown. (See Sections 4.1 *General Notes* and 4.2 *RNA Reverse Transcription Notes*).

	Component	*Volume*	*Final Concentration*
Master Mix	$MgCl_2$ Solution	4 µL	5 mM
	10X PCR Buffer II	2 µL	1X
	Sterile Distilled Water	1 µL	—
	dGTP	2 µL	1 mM
	dATP	2 µL	1 mM
	dTTP	2 µL	1 mM
	dCTP	2 µL	1 mM
	RNase Inhibitor	1 µL	1 U/µL
	Reverse Transcriptase	1 µL	2.5 U/µL
	Random Hexamers -or- Oligo $d(T)_{16}$ -or- "Downstream" Primer	1 µL	2.5 µM -or- 2.5 µM -or- 0.75 µM
	Positive Control RNA -or- Experimental Sample	2 µL	10^4 copies -or- ≤ 1 µg total RNA
	Total volume per sample	20 µL	

1.2 To reduce evaporation or refluxing, overlay the mix with 50 to 100 µL of mineral oil **(Part No. 0186-2302)**. The oil should not interfere when withdrawing samples. If the entire volume is to be recovered, 100 µL of high purity chloroform should be added after amplification. The aqueous phase containing the DNA will then float on the chloroform-oil mixture, allowing easy collection.

If using random hexamers, allow all tubes to incubate at room temperature for 10 minutes. The room temperature incubation allows for the extension of the hexameric primers by reverse transcriptase. The extended hexameric primers will then remain annealed to the RNA template upon raising the reaction temperature to 42°C. Incubate all tubes in a Perkin-Elmer Cetus DNA Thermal Cycler at 42°C for 15 minutes, 99°C for 5 minutes, and then soak at 5° for 5 minutes. Program a STEP-CYCLE as follows:

Segment 1:	42°C	15 minutes
Segment 2:	99°C	5 minutes
Segment 3:	5°C	5 minutes
Cycle Count:	1	

2.0 PCR Protocol

2.1 For each sample prepare a minimum of 78 µL of PCR Master Mix as shown below. (See Sections 4.1 *General Notes* and 4.3 *PCR Amplification Notes*).

Component	*Volume*	*Final Concentration*
$MgCl_2$ Solution	4 µL	2 mM
10X PCR Buffer II	8 µL	1X
Sterile Distilled Water	65.5 µL	—
AmpliTaq DNA Polymerase	0.5 µL	2.5 U/100 µL
Total volume per sample	78 µL	

2.2 Dispense 78 µL of the PCR Master Mix into each reverse transcription reaction tube. Change tips between additions to avoid sample carryover.

2.3 Dispense the primers into each tube. Change tips between primer additions to avoid sample carryover. For the Positive Control RNA supplied with this kit, use the DM151/DM152 primer set. If a "downstream" primer was used for the reverse transcription reaction (e.g., DM152), then it should not be added again. Instead, substitute 1 µL sterile distilled water.

Component	*Volume*	*Final Concentration*
"Downstream" Primer	1 µL	0.15 µM
"Upstream" Primer	1 µL	0.15 µM

The final volume of each tube should now be 100 µL (including 20µL from the reverse transcription reaction).

2.4 Spin the tubes for approximately 30 seconds in a microcentrifuge.

3.0 Temperature Cycling for the Control Reagents

Optimum performance of the *GeneAmp*™ PCR Process is achieved using the Perkin-Elmer Cetus DNA Thermal Cycler. For amplification of the Positive Control RNA using the *GeneAmp*® RNA PCR Kit, program the Perkin-Elmer Cetus DNA Thermal Cycler for 4 linked files as follows:

STEP-CYCLE:	2 minutes at 95°C for 1 cycle.
STEP-CYCLE:	1 minute at 95°C and 1 minute at 60°C for 35 cycles.
STEP-CYCLE:	7 minutes at 60°C for 1 cycle.
SOAK:	4°C

Refer to Section 4.3 *PCR Amplification Notes* and the DNA Thermal Cycler User Manual for additional programming instructions.

The PCR amplified samples can be stored frozen until subsequent analysis.

4.0 Notes

4.1 *General*

a. Due to the enormous amplification possible with the *GeneAmp* PCR process, small levels of DNA contamination, especially from previous PCR amplification reactions and Positive Control templates, can result in product even in the absence of purposefully added template DNA.[12] If possible, all reaction mixes should be set up in an area separate from PCR product analysis and sample preparation. Dedicated pipettes (preferably positive displacement pipettes), vessels and solutions for RNA/DNA preparation, reaction mixing, and sample analysis will minimize cross contamination.

b. Mix gently (avoid generating bubbles) the Reverse Transcriptase, RNase Inhibitor, and *AmpliTaq*® DNA Polymerase as well as other recently thawed reagents, then spin down in a microcentrifuge before pipetting. Pipette enzymes and RNase Inhibitor carefully and slowly: the viscosity of the 50% glycerol in the buffer can lead to pipetting errors. If possible, use a positive displacement pipette.

c. For both reverse transcription and PCR amplification, master mixes of reagents (water, buffer, dNTPs, magnesium chloride, and enzyme) for all samples can be prepared first, then aliquoted to individual tubes when needed. Using such mixes will minimize reagent pipetting losses, increase accuracy, and reduce the number of reagent transfers. Any combination of sterile distilled water, primer, and experimental sample volumes can be used as long as the final total combined volume equals 20 µL for the reverse transcription reaction (See Section 1.1.) and 100 µL for the PCR amplification (See Sections 2.1 and 2.3).

d. Reverse transcription and PCR amplifications are performed in capped 0.5 mL polypropylene microcentrifuge tubes. Since nucleases are often found on surfaces, it may be preferable to use sterile, siliconized tubes and pipette tips. Perkin-Elmer Cetus 0.5 mL *GeneAmp* Reaction Tubes **(Part No. N801-0180)** provide the best heat transfer when using the Perkin-Elmer Cetus DNA Thermal Cycler, because of their uniform fit in the wells.

e. The reagent concentration ranges in the reaction mix recipe described in **Sections 1.0 and 2.0** are a useful starting place for reverse transcription and PCR amplification of different RNA targets using primers designed by the user. Optimization of reactions for each primer-template pair may be necessary and can be achieved by varying magnesium chloride concentration, primer concentration and anneal-extend temperature **(Section 3.0)**. The effect of these variations can be monitored by examining the intensity and distribution of product samples electrophoresed on 3% NuSieve®/1% SeaKem® agarose (FMC Corp, Rockland, ME) gels and visualized with ethidium bromide staining.[9]

f. The optimal magnesium chloride concentration for both reverse transcription and PCR amplification needs to be determined empirically, by testing concentrations of magnesium chloride from 0.8 to 5 mM in 0.5 mM increments for each primer set. Too little or too much could reduce reverse transcription and/or amplification efficiency or result in non-specific products. If the samples contain EDTA or other chelators, raise the magnesium chloride concentration in the reaction mix proportionately. Magnesium chloride concentrations should also be adjusted in parallel with significant changes in the concentrations (higher or lower) of sample RNA, cDNA, DNA, and dNTPs.

g. Keep concentrations of dNTPs in the reaction mix balanced; if the concentration of any one of dNTP is significantly different from the rest, the *AmpliTaq* DNA Polymerase will tend to misincorporate them, slow down and terminate prematurely.[13]

h. The sequence-specific primers for RNA and cDNA should be typically 15 to 30 bases in length. The %G+C of primers should be near 50% to maximize specificity. To avoid potential problems, primers should be purified by gel electrophoresis or HPLC ion-exchange chromatography. The optimal primer concentrations need to be determined empirically, by testing concentrations in the range of 0.1 to 1.0 µM. Primer concentrations that are too low will result in little or no PCR product, while concentrations that are too high may result in amplification of non-target sequences. Primer concentrations in the range of 0.2 to 0.5 µM will work for most PCR amplifications. Primer sequences should not complement within themselves or to each other, particularly at the 3' end.

4.2 *RNA Reverse Transcription*

a. The primer for cDNA synthesis can be Random Hexamers, Oligo $d(T)_{16}$ [if poly (rA)-tailed mRNA is used], or a "downstream" primer of your own design (see "Primer Sequences" section for selection of primer to use). The cDNA synthesis primer for the Positive Control RNA can be Random Hexamers, Oligo $d(T)_{16}$, or the DM152 "downstream" primer.

b. The RNA segment to be transcribed and later amplified can be at least 3 kb long, although 100 to 1000 bases are more typical and easier to amplify. Start with enough copies of the template to be sure of obtaining a signal after 35 cycles: preferably $>10^4$ copies but less than 1 μg total sample RNA. Low concentrations of target cDNA may require up to 45-50 cycles to produce sufficient product for analysis.

c. Sample RNA should be extracted with phenol/chloroform, precipitated with ethanol, and redissolved in RNase-free 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl, pH 8.0.[11]

d. ***Important:*** Reverse transcriptase binds to cDNA and is thereby inhibitory to PCR amplification. Under the conditions provided (2.5 U/μL), the 99°C incubation for 5 minutes inactivates the reverse transcriptase and removes the inhibitory effect. If the concentration of reverse transcriptase is increased, inactivation of reverse transcriptase becomes more difficult. At 10 U/μL of reverse transcriptase, PCR yields may be variable or sporadic. Therefore, for synthesis of longer RNA trancripts, it is advisable to increase the incubation time during reverse transcription up to 60 minutes rather than increase the amount of Reverse Transcriptase added.

4.3 *PCR Amplification*

a. The selection of 60°C for the anneal-extend temperature is optimal for amplification of the positive control cDNA. It may be necessary to lower or raise (in the range of 37° to 65°C) the anneal-extend temperature for other primer-template pairs. Higher anneal-extend temperatures generally result in a much more specific product[8]; the optimal anneal-extend temperature can be determined empirically by testing increments of 5°C or less until the maximum in specificity and product yield is reached. At these temperatures (37°C to 65°C) *AmpliTaq*® DNA Polymerase has significant activity and extension of primed templates can occur. For some primer-template pairs, extension is completed during the 1 minute incubation at 37° to 65°C, so no hold time at 72°C is necessary.

b. The length of the target sequence will affect the required extension time. Typically, *AmpliTaq* DNA Polymerase has an extension rate of 2,000 to 4,000 bases per minute at 70° to 80°C. Polymerization rates are significant even below 55°C and, with some templates, up to 85°C.[12] As the amount of DNA increases in later cycles, the number of *AmpliTaq* DNA Polymerase molecules may become limiting for the extension time allotted. Increasing the extend times in later cycles may be necessary to maintain efficiency of amplification. This can be achieved by using the Auto-Segment Extension feature of the Perkin-Elmer Cetus DNA Thermal Cycler.

c. The half life of *AmpliTaq* DNA Polymerase (<35 minutes at >95°C)[14] suggests 95°C as the maximum practical melting temperature. It is very important in the early cycles to be sure to completely melt the template DNA. With high G+C content DNA, melting at 97°C for the first few cycles will help produce single stranded template for the PCR reaction. The melting temperature **should be reduced** for subsequent cycles because the smaller PCR product usually melts completely at a lower temperature (unless the PCR product is excessively G+C rich) than the starting DNA.

PERFORMANCE CLAIMS

Under the conditions described above, the *GeneAmp*® RNA PCR Kit when used with the DNA Thermal Cycler will:

- yield a visible 308-bp band on a 3% NuSieve®/1% SeaKem® agarose gel stained with ethidium bromide starting with 10^4 copies of Positive Control RNA.
- generate a cDNA product in excess of 2 Kb.

PRIMER SEQUENCES

The sequences of the RNA and PCR primers provided in this kit are shown below.

Random Hexamers	Random distribution of hexameric oligonucleotides containing all possible combinations of G, A, T, and C.
Oligo $d(T)_{16}$	5′-TTTTTTTTTTTTTTTT-3′
DM151	5′-GTCTCTGAATCAGAAATCCTTCTATC-3′
DM152	5′-CATGTCAAATTTCACTGCTTCATCC-3′

The choice of primer for reverse transcription depends on many factors and the choice is best made on the basis of experimentation to evaluate all three priming systems. For short RNAs containing no hairpins, any of the three priming systems usually work equally well. For longer transcripts or sequences containing hairpins, the following guidelines should be considered.

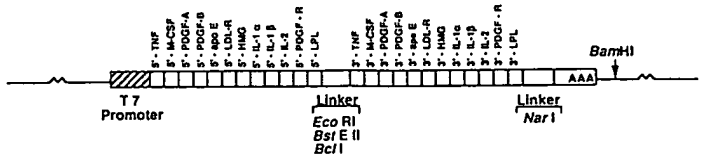
Random hexamers: Try first, especially if efficiency of downstream priming is low, for long reverse transcripts, or for reverse transcripts containing hairpins. Used to reverse transcribe all RNA (rRNA, mRNA, and tRNA). The concentration of random hexamers provided in this kit will allow efficient reverse transcription of at least 1 μg RNA.

"Downstream" Primer: Try second. Use to reverse transcribe only RNA that contains a complementary sequence. You will need to have prior knowledge of the target sequence and have to synthesize the primers.

Oligo $d(T)_{16}$: Avoid for long mRNA or large transcripts if target is several Kb upstream. Used to increase specificity by reverse transcribing only eukaryotic mRNAs and retroviruses with poly (rA)tails. [Prokaryotic RNA, rRNA, tRNA, and some eukaryotic mRNA do not have poly (rA) tails.] Used to transcribe entire eukaryotic mRNA message.

POSITIVE CONTROL RNA

The following schematic diagram (not drawn to scale) shows the general arrangement of the RNA transcribed from plasmid pAW109. The DNA sequences for the other primers in the control cDNA sequences are provided in Reference 1. The DM151 and DM152 primer set amplify a 420-bp sequence from authentic IL-1α mRNA.



Based on the positioning of the DM151/DM152 primer pair, a 308-bp product is amplified from pAW109-derived cDNA.

REFERENCES

1. Wang, A.M., Doyle, M.V., and Mark, D.F. 1989. Quantitation of mRNA by the polymerase chain reaction. Proc. Natl. Acad. Sci, USA. 86: pp. 9717-9721.

2. Kawasaki, E.S., and Wang, A.M. 1989. Detection of Gene Expression. In: Erlich, H.A. ed. PCR Technology: Principles and Applications of DNA Amplification. Stockton Press, Inc., New York, NY, pp. 89-97.

3. Wang, A.M., and Mark, D.F. 1990. Quantitative PCR. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. eds. PCR Protocols. A Guide to Methods and Applications. Academic Press, Inc., San Diego, CA, pp. 70-75.

4. Lee, F.S., and Vallee, B.L. 1989. Expression of Human Ribonuclease Inhibitor in *Escherichia coli*. Biochemical and Biophysical Research Communications 160: 115-120.

5. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., eds. 1990. PCR Protocols. A Guide to Methods and Applications. Academic Press, Inc., San Diego, CA.

6. Mullis, K.B., and Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. In: Wu, R. ed. Methods in Enzymology, vol. 155, Academic Press, San Diego, CA, pp. 335-350.

7. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

8. Scharf, S.J., Horn, G.T., and Erlich, H.A. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science 233: 1076-1078.

9. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

10. Lawyer, F.C., Stoffel, S., Saiki, R.K., Myambo, K.B., Drummond, R., and Gelfand, D.H., 1989. Isolation, charaterization, and expression in *Escherichia coli* of the DNA polymerase gene from the extreme thermophile, *Thermus aquaticus*. J. Biol. Chem. 264: 6427-6437.

11. Sambrook, J., Fritsch, E.F., and Maniatis, T., eds. 1989. Molecular Cloning: A Laboratory Manual. *2nd* ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 7.10- 7.11.

12. Higuchi, R., and Kwok, S. 1989. Avoiding false positives with PCR. Nature 339: 237-238.

13. Innis, M.A., Myambo, K.B., Gelfand, D.H., and Brow, M.A.D., 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Proc. Natl. Acad. Sci. USA. 85: 9436-9440.

14. Gelfand, D.H., and White, T.J. 1990. Thermostable DNA polymerases. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. eds. PCR Protocols. A Guide to Methods and Applications. Academic Press, Inc., San Diego, CA., pp. 129-141.

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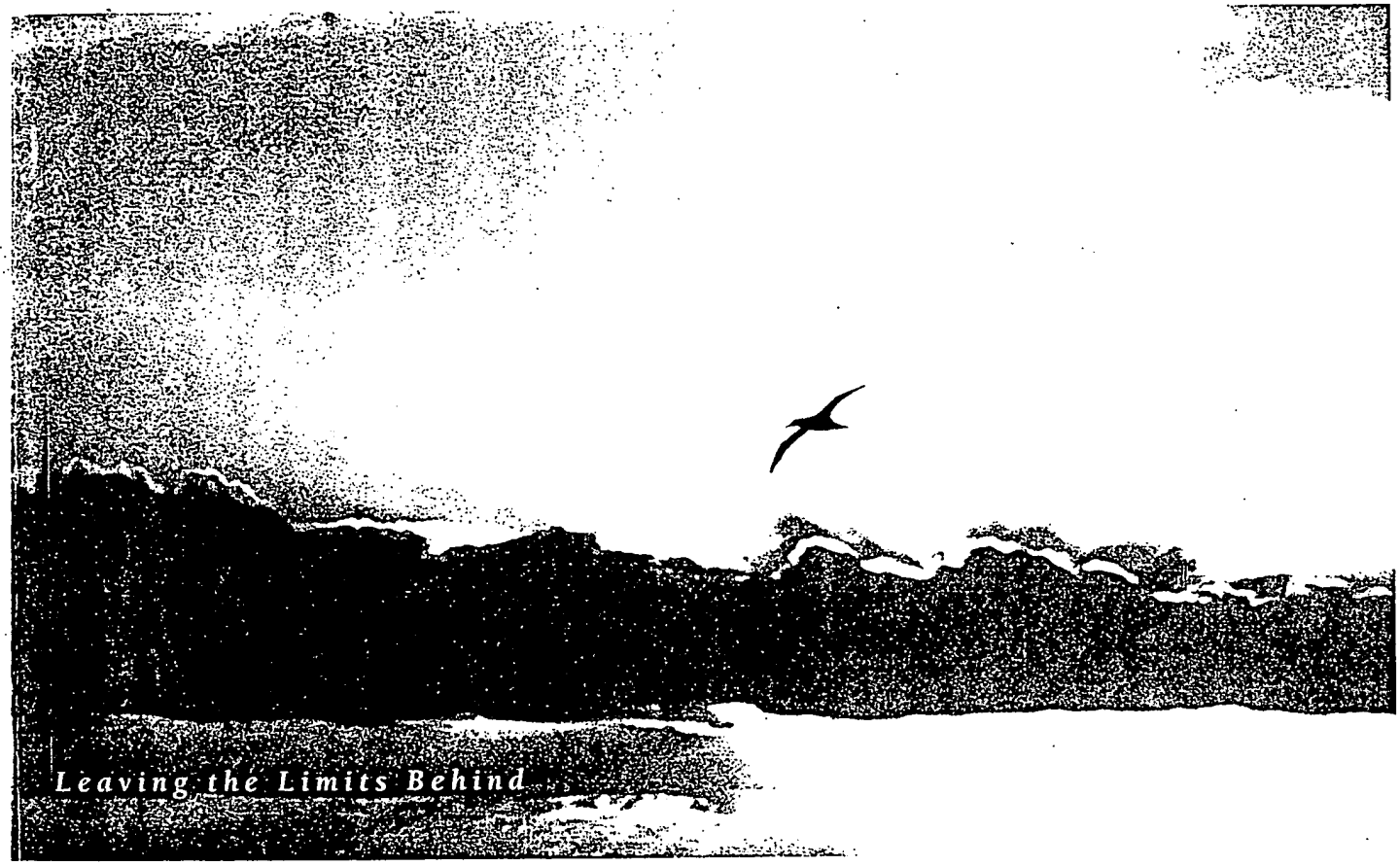

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Molecular Biology
The disc-like adhesive pads on the hands and toes of the tree frog enable this nimble creature to perform the most sensitive acrobatic maneuvers. Likewise, the Boehringer Mannheim "Genius" System makes sensitive scientific procedures equally as swift and safe.
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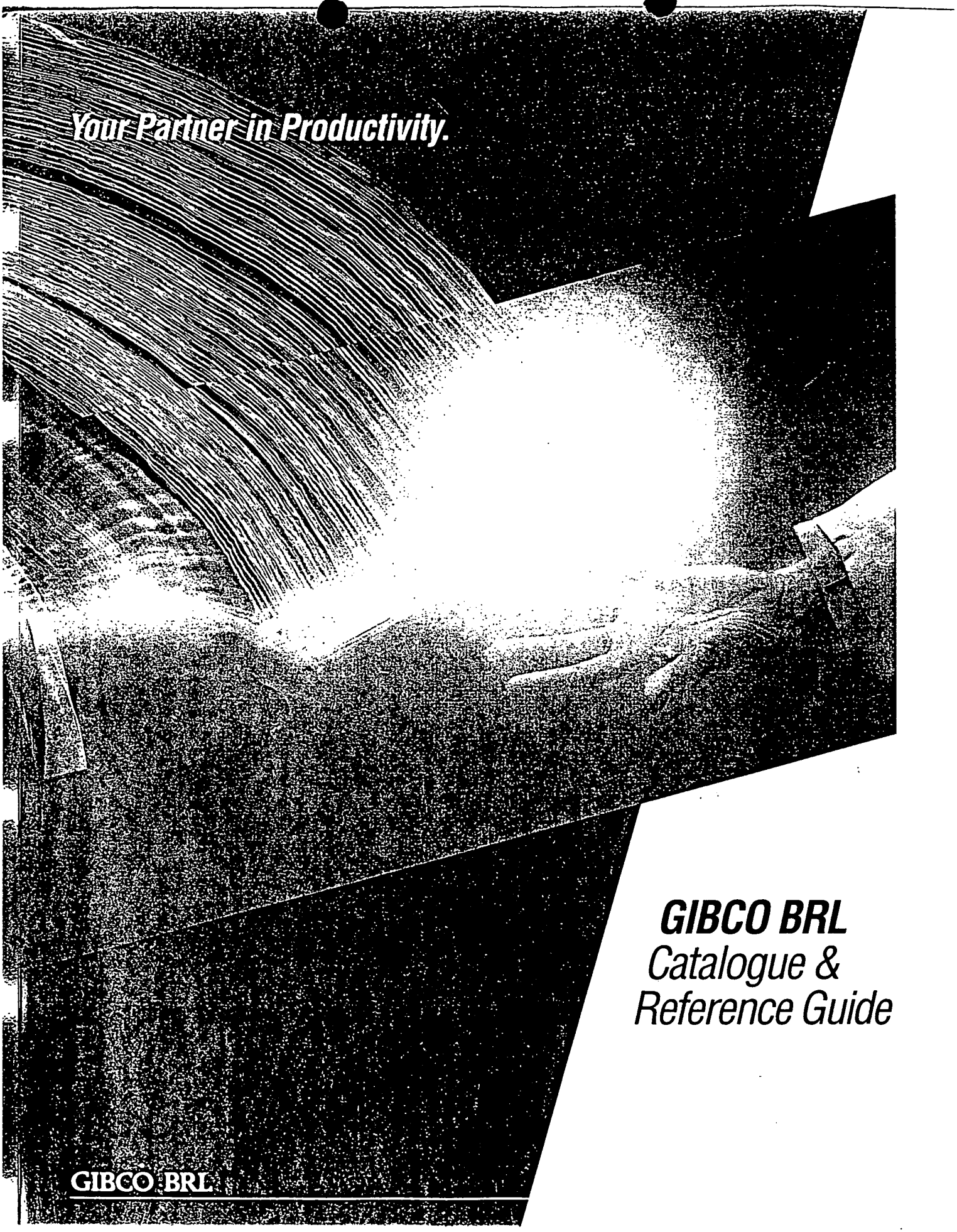

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CHAPTER 7

Enzymes

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STRATAGENE

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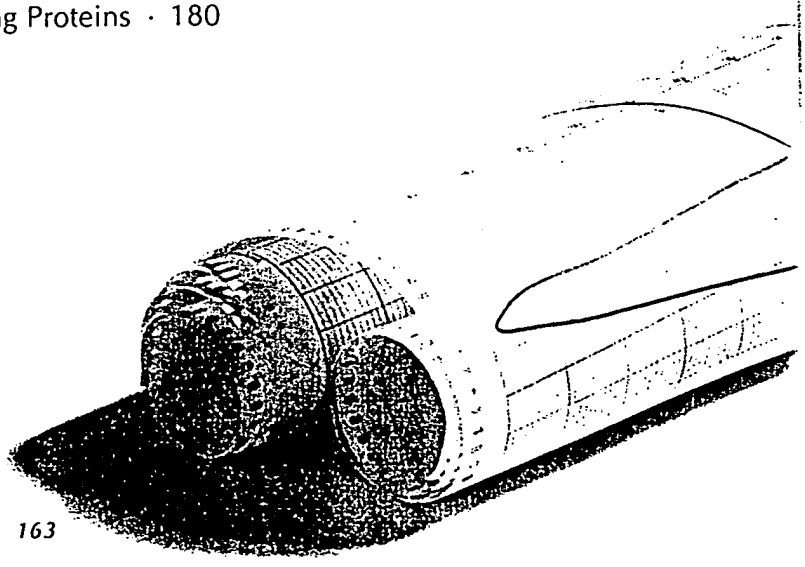
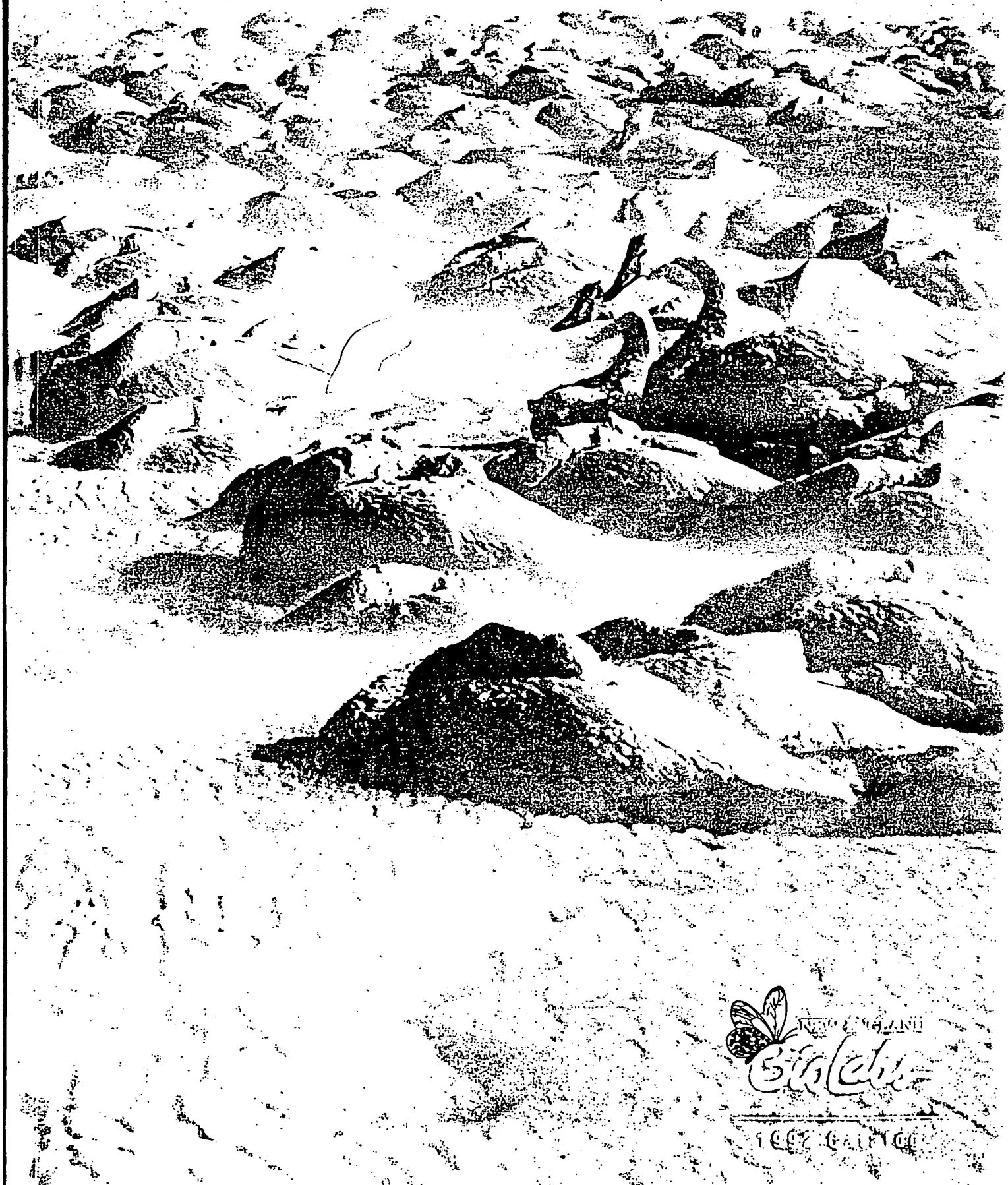
Creating the tools for the creative mind

STRATAGENE

Modifying Enzymes





Applications of Modifying Enzymes

	E. coli DNA Polymerase I	Klenow	Vent™ DNA Polymerase	Vent™ (exo–) DNA Polymerase	Deep Vent™ DNA Polymerase	T4 DNA Polymerase	T7 DNA Polymerase	M-MuLV Reverse Transcriptase	T7 RNA Polymerase	SP6 RNA Polymerase	T4 DNA Ligase	*E. coli* DNA Ligase	T4 RNA Ligase	Bal-31 Nuclease	Exo III Nuclease	Mung Bean Nuclease	T4 Polynucleotide Kinase	CIP
Labelling In vitro																		
5′																	+	
3′ addition													+					
3′ fill-in	(+)	+	+	+	+	+	(+)											
3′ exchange	(+)	(+)	+		+	+	(+)											
nick-translation	+																	
replacement synthesis	(+)	(+)	(+)		(+)	+									+			
random priming		+	+	+	+													
RNA probes									+	+								
Cloning																		
creating blunt ends																		
5′ overhang fill-in	(+)	+	+		+	(+)	(+)											
3′ overhang removal	(+)	(+)	+		(+)	+	+									+		
ligation																		
blunt-ended duplex											+							
cohesive-ended duplex											+	+						
single-stranded													+					
nicks											+	+						
phosphorylation																		
5′																	+	
dephosphorylation																		
3′																	+	+
5′																		+
nested deletions						+								+	+	+		
Sequencing																		
DNA		+		+				(+)							+			
RNA								+										
Mutagenesis																		
oligonucleotide directed		(+)	+		+	+	(+)											
misincorporation		+													+			
Mapping																		
restriction														+	+			
footprinting															+			
transcript								+								+		
cDNA																		
synthesis																		
1st strand								+										
2nd strand	(+)	+	(+)		(+)													
cloning																		
Okayama-Berg								+				+						
In vitro transcription									+	+								

\+ = recommended enzyme for this application
(+) = usable, but not the enzyme of choice